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A Non-coding RNA of Insect *Hz*NV-1 Virus Establishes Latent Viral Infection through MicroRNA

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Heliothis zea **nudivirus-1 (*Hz*NV-1) is an insect virus previously known as *Hz*-1 baculovirus. One of its major early genes, *hhi1*, is responsible for the establishment of productive viral infection; another gene, *pag1*, which expresses a non-coding RNA, is the only viral transcript detectable during viral latency. Here we showed that this non-coding RNA was further processed into at least two distinct miRNAs, which targeted and degraded *hhi1* transcript. This is a result strikingly similar to a recent report that herpes simplex virus produces tightly-regulated latent specific miRNAs to silence its own key early transcripts. Nevertheless, proof for the establishment of viral latency by miRNA is still lacking. We further showed that *Hz*NV-1 latency could be directly induced by *pag1*-derived miRNAs in cells infected with a *pag1*-deleted, latency-deficient virus. This result suggests the existence of a novel mechanism, where miRNAs can be functional for the establishment of viral latency.**

Heliothis zea nudivirus 1 (*Hz*NV-1) is a baculiform insect virus with a circular double-stranded DNA genome. This viral genome has been sequenced and was found to encode approximately 154 open reading frames[1–5]. This virus was originally regarded as a member of the Baculoviridae family, but due to its lack of an occlusion body and low sequence homology to baculoviruses[6], it is now temporarily re-classified with other non-occluded viruses as a new *Nudivirus* genus[7–8]. The *Hz*NV-1 virus has a relatively broad host range and has been reported to infect many insect cell lines[1,9–11].

*Hz*NV-1 virus can establish distinct latent and productive infection cycles in insect cell cultures[3,12–14]. It thus provides a convenient model system for the study of molecular switches between these two viral infection cycles. During productive infection, the virus generates more than 100 transcripts, and high titers of virus progeny are produced. In this infection stage, the majority of Tn-368 and Sf-21 insect host cells are killed leaving a small percentage of the cells become latently infected[3,13]. During viral latency, the persistency-associated gene 1 (*pag1*), is the only viral gene transcript detected[4]. In this infection stage, virus genomes exist either as episomes or are inserted into the host genome, and persist through many cell passages without the releasing of viral particles[14]. Occasionally, viruses can be released from very small proportions of latently-infected cells (usually less than 0.2%), resulting in the death of these cells. This small quantity of continuously-released virions results in the presence of low viral titers (around 10^3 pfu/ml) in the culture medium of the latently-infected cells[4,14].

Although *pag1* is the only transcript detectable during latent viral infection, it is also expressed during productive viral infection. Uniquely, the transcript of *pag1* is a non-coding RNA, previously also referred to as the persistency-associated transcript (PAT1)[3–4], and was found to be involved in the establishment of latent *Hz*NV-1 virus infection[3–4]. The *pag1* has several interesting features. Sequence analysis of *pag1* revealed abundant direct and inverted repeats. The transcript of *pag1* is not translated, as it does not associate with polysome[4–5], and was later shown to be a nuclear RNA[4–5]. Although we can not rule out the possibility that *pag1* transcript is an intron of a larger transcript, the fact that the direct upstream sequence of *pag1* coding region is a promoter suggesting that this is more likely to be an independent transcript[3,5].

During productive viral infection, an abundant 6.2 kb transcript is expressed from a gene located in the fragment of *Hz*NV-1 *Hind* III-I (*hhi1*) of the viral genome[3–4,15–16]. *hhi1* is a viral gene expressed very early during viral productive infection (0.5 h post viral infection, hpi), and it was shown to involve in viral re-activation from latency[3,16]. It was also shown that the suppression of *hhi1* expression can switch viral infection from productive to latent infection[3,16]. In contrast with the expression of most immediate-early genes of baculovirus, the proper expression of *hhi1* requires viral factors[15].

Recently the herpes simplex virus (HSV-1) was shown to use the miRNAs derived from a latent transcript to block the function of early genes, without the proper function of early transcripts, it is speculated that virus may enter latency[17], however, a proof is still lacking. In this study, we found that *Hz*NV-1 can serve as a convenient system to investigate such possibility. We provided evidences showing that the *pag1* is capable of down-regulating *hhi1* transcript in this virus. Subsequent experiments showed that *pag1* functions through the expression of two microRNAs (miRNAs), which target to and degrade *hhi1* transcript. These miRNAs could be detected in viral infected cells and our data indicated that the miRNA derived from *pag1* can establish latent viral infection in the cells.

The establishment of latent viral infection through miRNA-producing non-coding viral RNA represents a simple yet highly effective way for *Hz*NV-1 to achieve latent viral infection in the host cells. Although different from *Hz*NV-1, a further prove for the miRNA to establish viral latency in HSV-1 may be still needed, the similarity between HSV and *Hz*NV-1 in using miRNA derived from latent-specific transcripts to control early transcript, and subsequently result viral latency represents an interesting viral convergent evolution. Furthermore, miRNA as a switch for the transition of productive to latent viral infections may be a widely-spreaded phenomenon of virus/host interactions in nature.

Results

***hhi1* expression is negatively regulated by *pag1*.** In order to study the interactions of *pag1* and *hhi1*, two expression plasmids, pKSpP1 and pKShHI, were constructed. The former used *pag1* and the latter used *hsp 70* promoters to express *pag1* and *hhi1* transcripts, respectively. Northern analysis showed that *pag1* was not only expressed in the productive and latent viral infected cells, it was also properly expressed in pKSpP1 transfected cells (Fig. 1A). Both plasmids were then co-transfected into SF-21 cells to identify any possible interactions. The *hhi1*-expressing vector pKShH1 was also transfected into SF-21 cells with an empty vector (pBluescript II KS−, Stratagene) as a control. Northern blot analysis showed that the level of *hhi1* expression decreased dramatically in the presence of *pag1* (Fig. 1B). To confirm that *pag1* can negatively down-regulate *hhi1* expression during *Hz*NV-1 infection, cells were first transfected with *pag1*-expression vector pKSpP1 and the transfected cells were subsequently infected with *Hz*NV-1. Similarly, *hhi1* expression from viral infection was suppressed by the transfection of pKSpP1 (Fig. 1C). In this experiment, the level of *hhi1* transcript was detected at 2 hours post *Hz*NV-1 infection (hpi), because, according to our previous study, this is the timing at which *hhi1* expression reaches to the highest level[16].

Prediction of possible *pag1* miRNA precursors. Previously, sequence analysis of *pag1* revealed the presence of abundant direct and inverted repeats with no significant open reading frames (ORFs) in its coding region[4–5]. In this experiment, our analysis showed that *pag1* transcript contains abundant stem-loop structures. Therefore, it is possible that *pag1* transcript may produce miRNAs to suppress the expression of *hhi1*. In order to explore this possibility, 23 possible miRNAs were first predicted from *pag1* coding region and five of them were predicted to target to *hhi1* coding region (Fig. 2).

Proper expression of miRNAs from *pag1* is confirmed in productively and latently infected cells, and also in cells transfected with *pag1* plasmid. To confirm whether the above predicted miRNAs truly come from *pag1* and are expressed in infected Sf-21 cells, a stem-loop PCR[18–19] was performed to analyze the miRNA expression in *pag1*-transfected cells. The *pag1*-transfected cells were harvested at 12 hpi and total RNA was extracted. The cDNAs of miRNAs were obtained using miRNA stem-loop RT primers (Table 1). Stem-loop PCR analysis was performed using these cDNAs as the templates with specific miRNA primers designed based on our predictions. In order to investigate whether if any of the predicted miRNAs are expressed in viral infected cells, total RNAs were extracted from productive and latent viral infected cells and subsequently analyzed by stem-loop PCR. Real-time PCR products were also separated by gel electrophoresis and visualized by EtBr staining as a further control to show proper expression of different PCR products (Fig. 3 and Supplemental Fig.1). The result showed that two miRNAs, hv-miR-246 5P and hv-miR-2959 5p, were expressed in the *Hz*NV-1 productively infected cells (Fig. 3A), *Hz*NV-1 latently infected cells (Fig. 3B), and *pag1*-transfected cells (Fig. 3C). These two miRNAs were cloned and the predicted secondary structures of hv-miR-246 5P and hv-miR-2959 5p precursors are shown in Fig. 3D, and Fig. 3E, respectively. Mature miRNA are shadowed in red and the numbers on nucleotides indicating their corresponding positions to *pag1* transcript.

The melting curve for hv-miR-246 5p had two peaks in the *Hz*NV-1 productively infected cells (Fig. 3A). We cloned these two fragments into cloning vector and analyzed their sequences. The sequence of the upper band did not match to the predicted miRNA and therefore is a non-specific product. The molecule size of the upper band is also lager than the predicted miRNA. In these experiments, proper expression of let-7a miRNA gene was performed as a positive control[20]. To further confirm the results from stem-loop PCR, northern blot analysis showed that hv-miR-246 and hv-miR-2959 became detectable in productively infected cells at 4 hpi and 8 hpi, respectively (Fig. 3F). However, they were not detected in mock infected cells (data not shown). These results indicated that the *pag1* transcript was indeed processed into miRNAs during the infection of *Hz*NV-1 (Fig. 3F).

***hhi1* expression can be down-regulated by *pag1* miRNAs.** To further test whether these two miRNAs target *hhi1* and suppress its transcripts, these miRNAs were separately transfected into Sf-21 cells along with a *hhi1*-expressing vector and the levels of *hhi1* transcript were analyzed by northern blot analysis at 12 hpi. Both miRNAs were able to reduce the amount of *hhi1* transcript within the cells (Fig. 4). The sequences of hv-miR-246 and hv-miR-2959 are mapped to nucleotides 226325 to 226366 and 226527 to 226555 of the *hhi1* coding region, respectively (Fig. 2). To confirm if the matching of miRNA sequences to *hhi1* was important, *i.e.*, acting as targeting sites, we constructed mutant miRNAs with three base-pair mutations in the matching regions for these two miRNAs (Fig. 4A, 4C). The mutational substituted bases are indicated by arrows. Northern analysis (Fig. 4B, 4D; left panels) and RT-PCR (Fig. 4B, 4D; right panels) showed that these two mutant miRNAs did not affect the levels of *hhi1* transcript (Fig 4B, 4D), suggesting that our predicted target positions are crucial for functioning. In these experiments, the levels of actin transcript were used as a loading control.

***pag1* miRNA functions in establishing latent viral infection.** Previously, we showed that *pag1* gene can promote the establishment of latent viral infection[4]. To test whether these miRNAs alone are enough to promote latent viral infection, Sf-21 cells were first transfected with miRNA followed by *Hz*NV-1 infection. The number of colonies was then recorded at 12 dpi. Most of the Sf-21 cells died when they were infected with *Hz*NV-1 virus alone and only a small percentage of cells became latently infected (Fig. 5A). However, the number of latently-infected cell colonies increased dramatically upon transfection of individual hv-miR-246 or hv-miR-2959 (Fig. 5B). We also analyzed the gene expression of *pag1* and *hhi1* in *Hz*NV-1 infected cells with or without miRNA treatments at 12 dpi (Fig. 5C). *hhi1* expression could only be

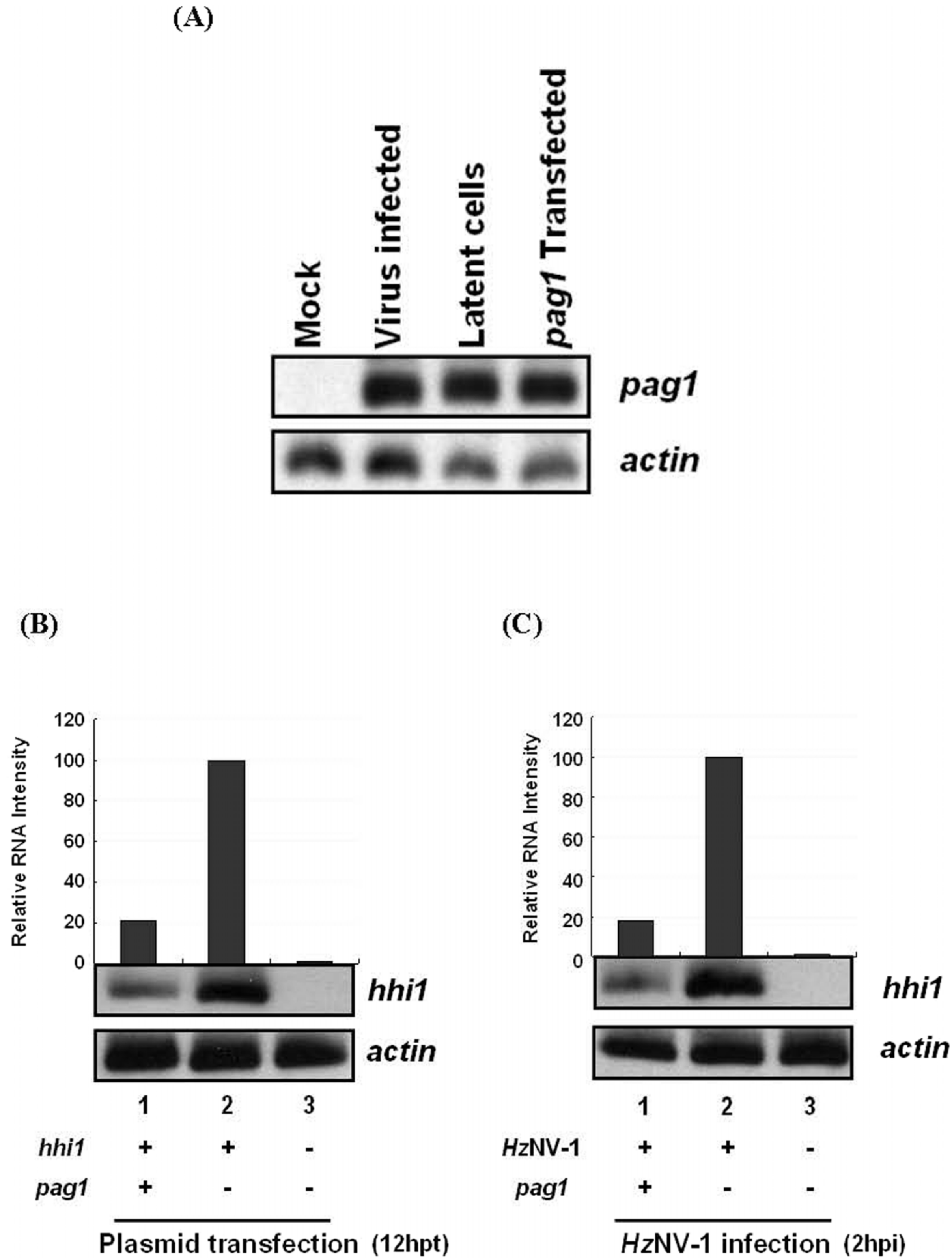

Figure 1 | Interactions of *hhi1* and *pag1* by northern analysis. (A) The expression of *pag1* transcripts in the productive infected, latent infected, and pKSpP1 transfected cells were probed. (B, C) pKShHI was either co-transfected with pKSpP1 (B), or co-infected with *Hz*NV-1 (C), then subjected to northern analysis using *hhi1* probe. Actin signal were used as a loading control.

detected in *Hz*NV-1 productively infected cells (Fig. 5C, lane 1) whereas *pag1* expression could be detected in productive and latent cells SFP4 (Fig. 5C, lanes 1 and 3). Cells transfected with these two miRNAs, hv-miR-246 and hv-miR-2959, produced only *pag1* transcript but not *hhi1* transcript, this is an evidence of viral latency (Fig. 5C, lanes 4 and 5). These results indicate that miRNAs alone can function to establish or at least to strongly promote latent viral infection. Furthermore, *pag1* transcript, but not that of *hhi1*,

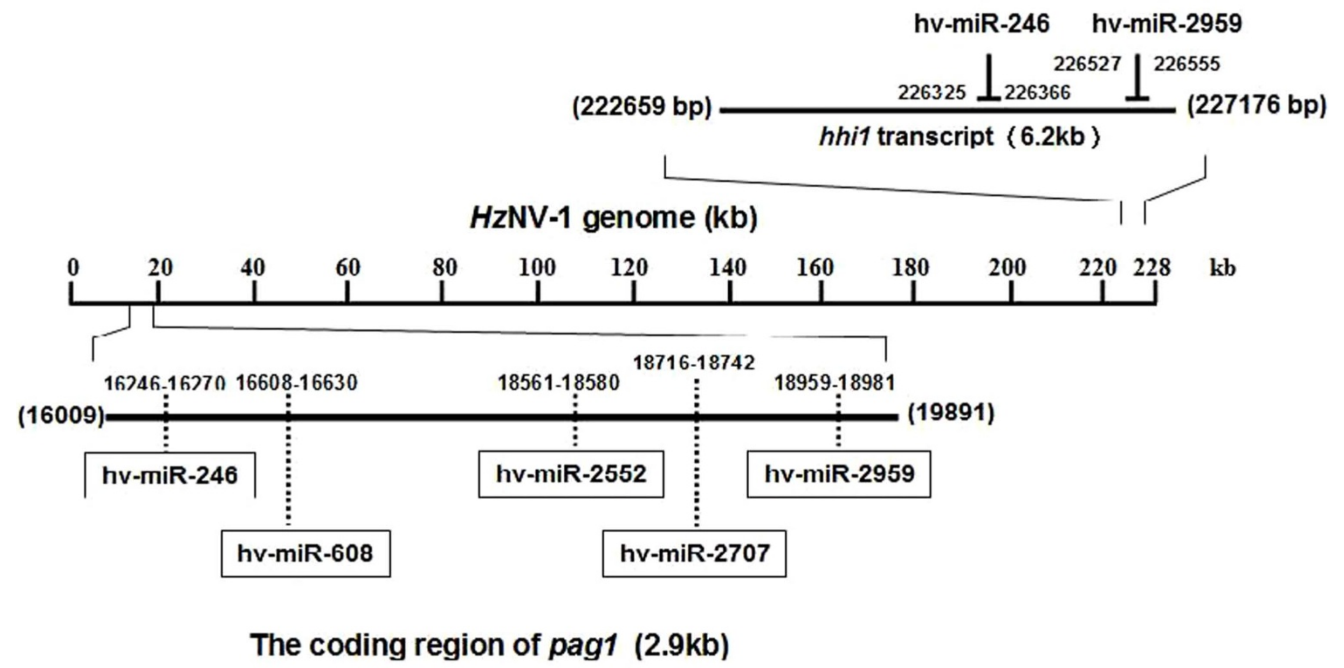

Figure 2 | Mapping of the predicted miRNAs in *pag1* transcript. Schematic drawing of the *pag1* transcript to show its 5 predicted miRNAs against *hhi1*, and 2 miRNAs, hv-miR-246 and hv-miR-2959, proved to be expressed by virus in the infected Sf21 cells.

was expressed in the latent cells induced by the transfection of miRNA. Besides, these latent cells can be cultured for long-term passages. All these evidences indicate strongly that the establishment of latent viral infection by miRNA is not a transient effect; it is a long term latent viral infection in the cells.

In these and previous experiments, we have observed that the number of latently-infected cell colonies increased upon miRNA or *pag1* transfection. However, whether these miRNA or *pag1* can only promote viral latency or are essential for the establishment of latent viral infection remain unknown. In order to confirm the function of the miRNA and *pag1*, we further constructed a *pag1*-null *Hz*NV-1 virus for infection experiment. Sf-21 cells were infected with *pag1*-null virus or transfected with a siRNA targeting to *pag1* transcript followed by *Hz*NV-1 infection. RT-PCR confirmed that the suppression of *pag1* gene expression by siRNA was successful (Fig. 6A) and also, no *pag1* expression was detected from *pag1*-null *Hz*NV-1 virus (Fig. 6B). Further experiments showed that upon knocking down (Fig. 6C, lane 5) or deletion of *pag1* transcripts (Fig. 6C, lane 1), the number of latent colony dropped significantly, and none of the cell colony can survive two weeks after viral infection. However, the number of latently-infected cell colonies increased dramatically upon transfection of individual hv-miR-246 or hv-miR-2959 (Fig. 6C, line 3, 4, 7 and 8). These cells keep on viable for long time (at least 2 months up to now). These experiments demonstrated that *pag1* and its derived miRNAs play a crucial role in the establishment of *Hz*NV-1 latent viral infection.

Table 1 | Oligonucleotides used in this study

microRNA name	primer name	sequence
hv-miR-246-3P	**2463pForward primer**	AACCGT**TTCTCGTCACGATAGCA**
	2463pUPL RT primer	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCAC**CAGAGCCAACACCATG**
hv-miR-246-5p	**2465pForward primer**	GGCTCC**AGGCTAAGCCCAGCT**
	2465pUPL RT primer	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCAC**CAGAGCCAACCTCGCC**
hv-miR-608-3P	**6083pForward primer**	GGTGGT**TCTCAAATTCAGAGCT**
	6083pUPL RT primer	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCAC**CAGAGCCAACATTACG**
hv-miR-608-5P	**6085pForward primer**	CAACGG**CTATGATGTCACGGTTTTG**
	6085pUPL RT primer	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCAC**CAGAGCCAACCCATGG**
hv-miR-2552-3P	**25523pForward primer**	TCCACA**CGTTCTGAAAACGACG**
	25523pUPL RT primer	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCAC**CAGAGCCAACTCTAAT**
hv-miR-2552-5P	**25525pForward primer**	TAACAC**CTTCTTAACTTATGTT**
	25525pUPL RT primer	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCAC**CAGAGCCAACTTTCTC**
hv-miR-2959-3P	**29593pForward primer**	AGAGGC**TACATGTAACAGCAATTGT**
	29593pUPL RT primer	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCAC**CAGAGCCAACAAAACA**
hv-miR-2959-5P	**29595pForward primer**	CACGTG**CGAGAACGGTTAATTGCA**
	29595pUPL RT primer	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCAC**CAGAGCCAACGATGCA**
hv-miR-2707-5P	**27075pForward primer**	GAGACA**CATGCTCATAGCGTAACG**
	27075pUPL RT primer	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCAC**CAGAGCCAACCAACAA**
let-7a	**7-Forward primer**	GCCGCTGAGGTAGTAGGTTGTA
	7-UPL RT primer	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCAC**CAGAGCCAACAACTAT**

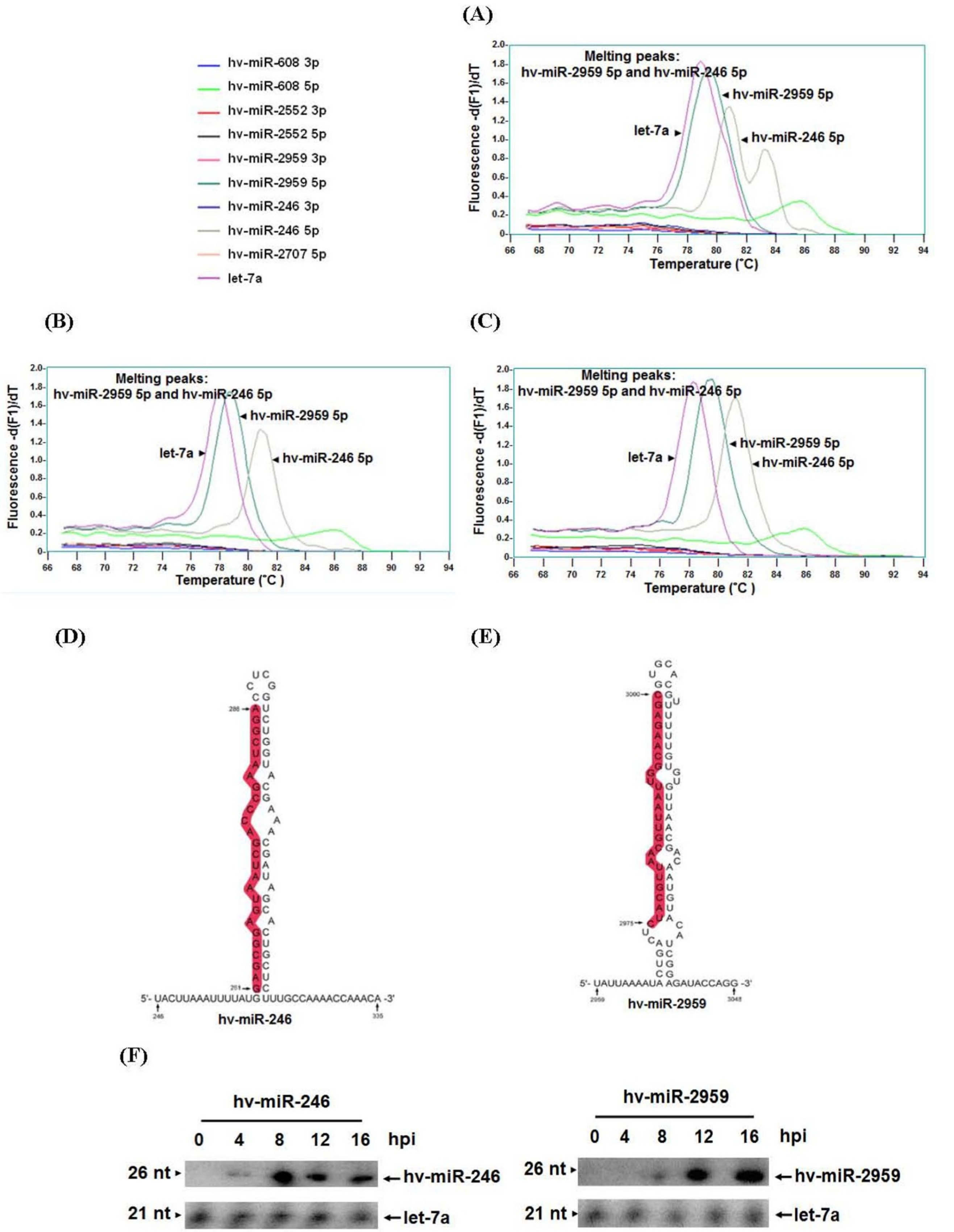

Figure 3 | Cloning and analysis of the predicted miRNA by stem-loop PCR and northern blot. Stem-loop PCR was performed to clone and analyze the proper expression of the predicted miRNAs in (A) *Hz*NV-1 productively infected cells, (B) *pag1*-transfected cells, and (C) *Hz*NV-1 latently infected cells. (D, E) Predicted secondary structures of hv-miR-246 5P (D), and hv-miR-2959 5p (E), precursors. (F) Small RNAs harvested from *Hz*NV-1 productively infected cells at various time points were analysis by northern blots with probes against predicted *Hz*NV-1 miRNAs (top panels) or let-7a miRNA as a positive control (bottom panels).

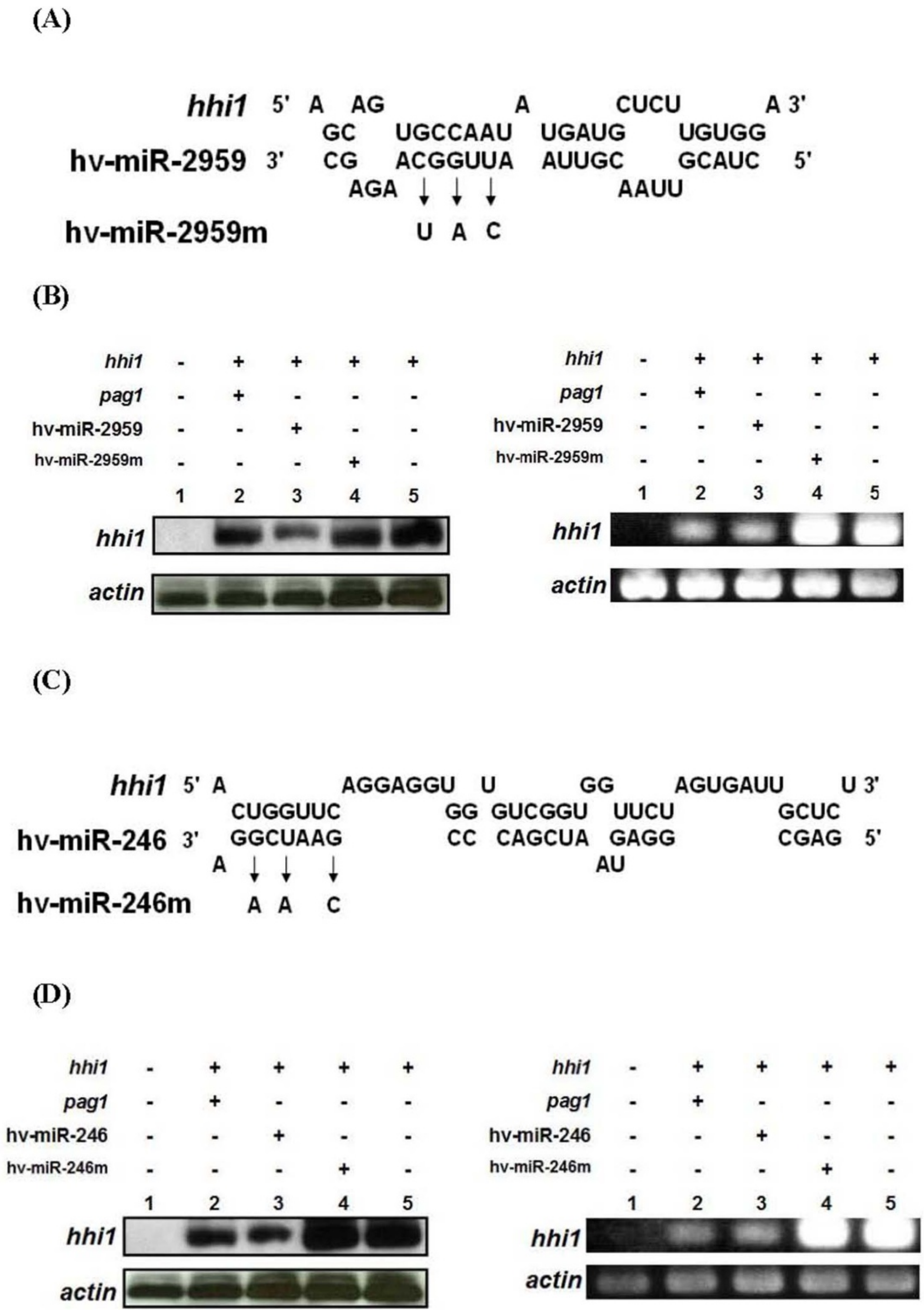

Figure 4 | Down-regulation of *hhi1* expression by *pag1*, hv-miR-246, and hv-miR-2959. (A, C) sequences of hv-miR-2959 and hv-miR-246 were shown, and miRNAs with mutations were denoted as hv-miR-246m and hv-miR-2959m, separately. (B, D) levels of *hhi1* transcript in various treatments were analyzed by northern hybridization (left panel) and RT-PCR (right panel).

Discussion

Previously, we found that *hhi1* can activate the expression of many early *Hz*NV-1 genes and reactivate *Hz*NV-1 virus from latency[16]. We had also discovered that the transcript of *pag1*, a non-coding RNA, plays a critical role to block *hhi1*-induced apoptosis[21] and to the establishment of latent viral infection[4]. In this study we found that *pag1* transcript could suppress *hhi1* expression remarkably through the targeting of *hhi1* transcript by miRNAs. Interestingly, these two miRNAs, hv-miR-246 or hv-miR-2959, were individually sufficient for strong promotion and/or establishment of latent viral infection. The observation that *pag1*-null *Hz*NV-1 virus was unable to establish latent infection further suggested that *pag1* plays a key role in the establishment of latent infection.

Most miRNA target sites have perfect pairing to the seed region located near the miRNA 5′ end. Nevertheless, recent reports have also demonstrated that perfect pairing between target sequence and the miRNA can also take place near the 3′ end of miRNA[22–23]. Our results also showed that the 3′ ends of miRNAs derived from *pag1* have perfect base pairings to the target sequences, and mutagenesis experiments showed that these pairing are critical for functioning. To further confirm that inhibition of *hhi1* expression was mediated through miRNA pathway, the effects of silencing *dicer1* and *ago1* expressions (Supplemental Fig. 2A) on the production of *pag1*-derived miRNA and control *let-7a* were assessed in *Drosophila* S2 cells (Supplemental Fig. 2B). It is known that *dicer1* is an important component of the miRNA progenesis; whereas, *ago1* is not involved

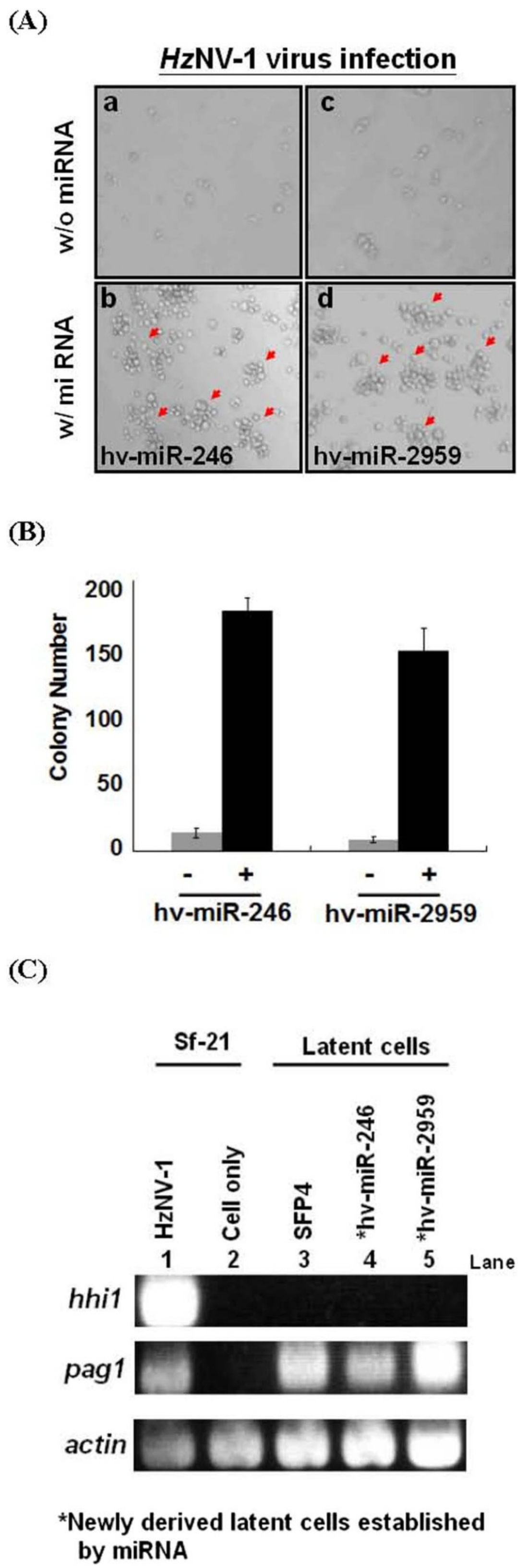

Figure 5 | Establishment of latent viral infection by miRNAs. (A) Sf21 cells were transfected with or without miRNA followed by *Hz*NV-1 infection. (B) Column representation of the results of panel (A). (C) Confirmation of *hhi1* and *pag1* expressions in various cells by RT-PCR.

in the generation of mature RNAs, rather, is involved in the interaction between miRNA and target transcript. Thus, it is reasonable that levels of miRNAs hv-miR-246, hv-miR-2959 and *let-7a* were not affected by *ago1* knockdown using stem-loop RT-PCR analysis (Supplemental Fig. 2B). We also found that *hhi1* expression was not down-regulated by *pag1* in either *dicer1* or *ago1* knocked down cells (Supplemental Fig. 2C), suggesting that miRNA pathway is essential for the production of *pag1*-derived miRNAs.

*Hz*NV1 virus is an insect-specific virus. In our studies, we found it shares striking similarity in the gene regulation networks during viral latency with the vertebrate virus, HSV-1[24]. Despite the high numbers of viral transcripts produced during productive viral infections, both viruses silence the expression of all genes except the expression of latency-associated transcript (LAT) in HSV-1[25], and *pag1* transcript in *Hz*NV-1[3–4], during latent viral infection. It is interesting to note that *Hz*NV-1 is a baculiform insect virus and HSV, on the other hand, is an icosahedra virus. They are quite distinct in shape and genomic sequences and are obviously not evolved from the same origin. Nevertheless, LAT is a latency-associated transcript, which contains a non-coding intron[25] and *pag1* transcript is similarly a non-coding transcript predominately expressed during viral latency[4]. Such striking similarity between these transcripts strongly suggests that the strategy for producing a non-coding RNA during latency is not an isolated viral infection phenomenon restricted to the HSV of vertebrates.

LAT was previously identified as an 8.3 kb transcript expressed from the genome of HSV[22]. This RNA is spliced and yields a stable intronic non-coding region of 2 kb and an unstable exonic RNA. This unstable exonic RNA was further processed to produce several miRNAs, which are found to suppress protein expression of early HSV genes, ICP0 and ICP4[17,26]. More recently, two sRNAs of 62 nt and 32 nt, but not miRNA, were predicted to be located on the 2 kb stable intronic region. Shen *et al.* (2009) found that these sRNAs play a role through an unidentified mechanism to inhibit apoptosis and productive infection[27]. Although these authors showed that these miRNAs or sRNAs were likely responsible for latent HSV infection due to the suppression of two important early genes, direct proof of latent establishment due to these miRNAs or sRNAs is still lacking.

In our results, due to *Hz*NV-1 can establish latent viral infection in insect cells, we were able to show that *Hz*NV-1 virus establishes latent viral infection through the suppression of the *hhi1* transcript using non-coding transcript *pag1*. We also showed that even miRNAs from *pag1* transcript alone are sufficient to establish latent viral infection. Since the transiently transfected miRNA is sufficient for the triggering of latent viral infection using *pag1*-null *Hz*NV-1, suggesting that once entering viral latency, viral gene expression is some how restricted without the need of a continuous supply of *pag1* transcript for *hhi1* suppression.

Our experiments showed that, although *Hz*NV-1 virus is not a herpes virus and *pag1* transcript has no sequence homology with LAT, shutting-off of all genes except the expression of one non-coding transcript during latency of these two viruses is a striking convergent evolution of viral-host interactions. In addition, the use of miRNAs derived from the non-coding RNA for similar functions by two unrelated viruses suggests that miRNA can be an important and effective mechanism for switching between productive and latent infections in a wide-range of host cells. Furthermore, *pag1* transcript is a stable non-coding RNA consistently observed during productive and latent viral infections[3–4], which resembles the intronic stable non-coding RNA of LAT. Although in the study of herpes virus, miRNA was not found from the stable non-coding RNA of LAT, we showed that *pag1* transcript can generate functional miRNAs.

In Fig. 7, we summarize the interactions between *hhi1* and *pag1* to better illustrate how they function as molecular switches to determine should the viruses enter productive or latent infections in the

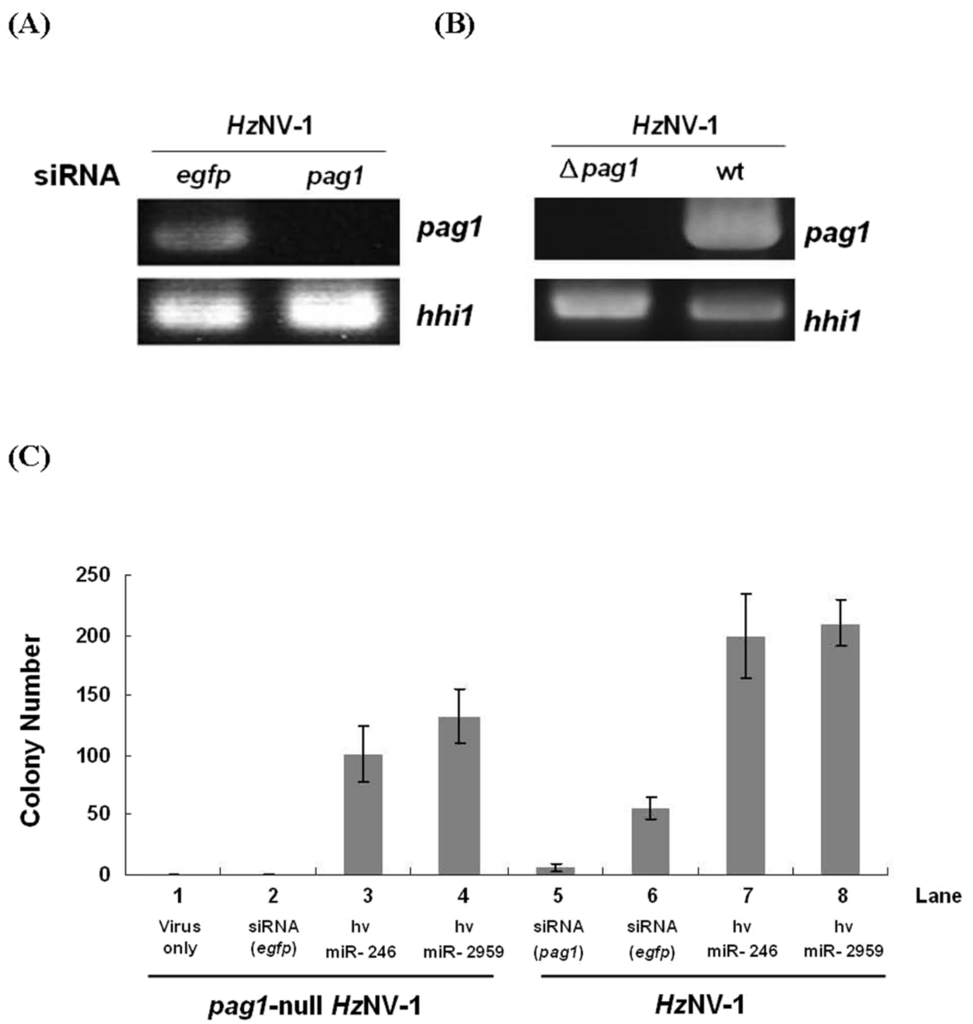

Figure 6 | Suppression of *Hz*NV-1 viral latency by knocking down *pag1* expression. (A) RT-PCR showed that artificial siRNA can efficiently suppress *pag1* expression in *Hz*NV-1 infected cells. (B) *pag1* expression is not detectable by RT-PCR in the *pag1*-null *Hz*NV-1-infected cells. (C) Formation of latent colony is not observed by the infection of *pag1*-null *Hz*NV-1.

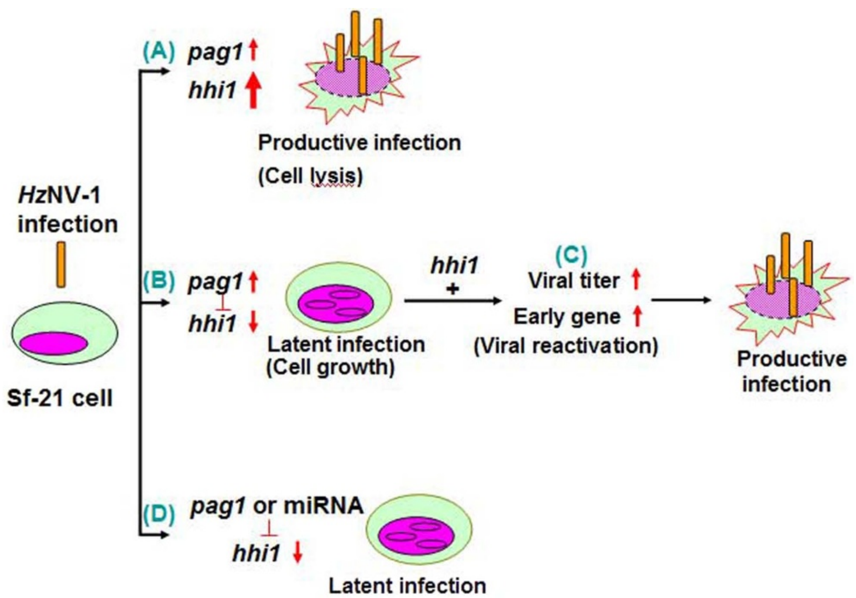

Figure 7 | A comprehensive model for the establishment of productive and latent *Hz*NV-1 viral infections through *hhi1* and *pag1* interactions.

host cells. The initial infection of *Hz*NV-1 results in high levels of *hhi1* expression (Fig. 5C, lane 1)[16], and a moderate level of *pag1* expressions (Fig. 5C, lane 1)[3–4]. The newly produced abundant *hhi1* transcript can tolerate some RNA degradation caused by the miRNAs derived from *pag1*. Then, the *hhi1* transcript, before degradation or survived from initial degradation, can quickly transactivate many viral early genes for productive viral infection to occur in most of the infected Sf21 cells (Fig. 7A)[16]. A low percentage of cells, by which *hhi1* transcript is successfully suppressed by *pag1* miRNA during initial viral infection, enter latency[14], where the level of *hhi1* transcript becomes undetectable while still maintain a moderate level of *pag1* transcript (Fig. 7B; Fig. 5C, lanes 3–5)[3]. Viral re-activation of latent cells can be induced by introducing *hhi1* transcript, which then stimulates HzNV-1 early genes expression, resulting in virus production (Fig. 7C)[16]. If *pag1* gene[4] or its derived miRNAs (Fig. 6C lanes 3,4) were transfected into the cells prior to the initial viral infection, the chance of cells entering latent viral infection becomes strongly boosted (Fig. 7D). Different from the function of miRNAs produced from LAT of the herpes virus, which blocks protein expression, miRNAs derived from *pag1* transcript degrade the transcript of *hhi1*. More significantly, our evidences proved that *pag1* transcript and its derived miRNAs can establish latent viral infection in the cells.

Methods

Cells and virus. *Spodoptera frugiperda* IPLB-Sf-21 was incubated in TC-100 insect cell culture medium, which contained 10% of FBS at 26°C (Gibco BRL)[28–29]. Standard *Hz*NV-l virus was derived by a serial dilution of the stock viral solution and isolated by plaque purification. Latently infected colonies were calculated as described in Wu *et al.*[16]. The titers of the virus clones were estimated by both Q-PCR[30] and $TCID_{50}$[28–29].

Plasmid construction. We obtained *pag1* and *hhi1* coding regions by PCR and inserted them down-stream to the *hsp 70* promoter (*p-hsp*)[31] and *pag1* promoter[16] of plasmids pKSh and pKSp, respectively to result plasmids pKShHI and pKSpP1. The nucleotide sequence of the *hhi1* gene was submitted to GenBANK, with an accession number of AF264019.

Computational prediction of viral miRNAs. The *pag1* miRNA prediction was carried out using the complete gene sequence of *pag1* (NC_004156) obtained from the National Center for Biotechnology Information. The *pag1* miRNA prediction was performed using miRNA prediction database (Vir-Mir) (http://alk.ibms.sinica.edu.tw)[32].

RNA isolation and northern blot analysis. 2×10^5 cells/well were seeded in a 24-well culture plate (Corning) and then transfected with two plasmids, pKShHI and pKSpP1, 0.5 μg each, separately or collectively, using Cellfectin according to the manufacturer's protocol (Invitrogen). Sf-21 cells were inoculated at a multiplicity of infection (MOI) of 1 with *Hz*NV-l virus and incubated at 26°C with gentle rocking. After absorption, total cellular RNA samples were extracted from productively-infected cells at the indicated time and from latently-infected SFP4 cells[3,16] using RNeasy Mini Kit (Qiagen). Briefly, total RNA were extracted from virus-infected Sf-21 or SFP4 cells (Qiagen). Then, equal amounts of RNA samples (15 μg each) were loaded, blotted onto Hybond-N+ nylon transfer membrane (GE Healthcare), and hybridized with *hhi1* or *pag1* specific probes, which were labeled with DIG by PCR amplification of template *Hz*NV-1 viral DNA using the PCR DIG Probe Synthesis Kit (Roche Applied Sciences) according to the manufacturer's instructions.

miRNA assay by stem-loop real-time PCR and northern blot. The total RNA samples from *pag1* transfected cells were isolated using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The miRNA cDNAs were synthesized from total RNAs using miRNA specific stem-loop primers (Table 1) according to criteria described previously[18]. Analysis of mature miRNA expression by RT–PCR was also carried out using a method described previously[18]. The cDNAs were diluted 10 times to perform PCR for expression confirmation and expression pattern analysis. Real-time PCRs were performed, respectively, in 20 μl mixture containing 1 μl cDNA, 0.5 μM forward and reverse primers, and 2 × SYBR Green qPCR master mix (Fermentas) with the following parameters: 94°C for 15 s, 60°C for 30 s and 72°C for 30 s. The real-time PCR products were detected by electrophoresis with 3% agarose gel containing ethidium and photographed under UV light. Expression of let-7a, a miRNA normally expressed in most of the cells[20], was used as a positive control.

The *pag1*-associated small RNA samples from *pag1* transfected cells at different time points were isolated using a mirVanaTM miRNA isolation kit (Ambion) according to the manufacturer's instructions. For northern blot analysis of small RNAs, 1 μg aliquots of each small RNA fraction, as well as radio-labeled Decade Markers (Ambion), were fractionated in 15% denaturing polyacrylamide gel electrophoresis (PAGE) gels (acrylamide : bis ratio, 19 : 1) containing 8 M urea in 0.5 × TBE buffer. The gels were soaked briefly in 0.2 μg/ml ethidium bromide in TBE to allow visualization of the RNA using a UV transilluminator (Bio-Rad, Hercules, CA). RNAs were transferred by electroblotting to a Hybond-N+ nylon transfer membrane (GE Healthcare) and UV cross-linked to the membrane. PAGE-purified DNA oligonucleotides (Integrated DNA Technologies) with the reverse complementary sequence to either candidate miRNAs or let-7a, were end labeled with [^{32}P]ATP (MP Biomedicals, Irvine, CA) to high specific activity. The labeled probes were purified using the *mir*Vana Probe & Marker kit (Ambion) according to the manufacturers' protocols. Hybridizations and washes were carried out using the ULTRAhyb-Oligo hybridization buffer according to the manufacturer's directions (Ambion).

Mutant miRNA experiments. 2×10^5 cells/well were seeded in a 24-well culture plate and then transfected the plasmid pKShHI (0.5μg) with hv-miR-246, hv-miR-246m, hv-miR-2959 and hv-miR-2959m (50nM each), separately, using Lipofectamine RNAiMAX (Invitrogen). Transfected cells were harvested at 12 hours post-transfection (hpt) for northern analysis using a *hhi1* specific probe. hv-miR-246m and hv-miR-2959m are three base mutants created in the perfect pairing regions of hv-miR-246 and hv-miR-2959, separately, to abolish the function of these miRNAs. All miRNAs duplex were synthesized by MDbio Inc. The miRNAs or mutant miRNAs used in this study are as follows:

hv-miR-246 (5′-AGGCUAAGCCCAGCUAAUGAGGCGAG-3′)
hv-miR-246m (5′-AGACAAACCCCAGCUAAUGAGGCGAG-3′)
hv-miR-2959 (5′-CGAGAACGGUUAAUUGCAAUUGCAUC-3′)
hv-miR-2959m (5′-CGAGAAUGAUCAAUUGCAAUUGCAUC-3′)

miRNA transfection upon *Hz*NV-1 infection. For *hhi1* and *pag1* miRNA transfected experiments, all miRNAs were synthesized by MDbio Inc. Sf-21 cells (4×10^4/well) in 96-well plates were transfected with 50 nM of the miRNA using Lipofectam RNAiMAX (Invitrogen). Cells were transfected with *pag1* miRNAs; then, at 4 hpt, the cells were infected with *Hz*NV-1 virus (MOI = 1), and the number of latently infected colonies was again calculated at 12 dpi[4]. Several primers were designed for the RT-PCR amplification of *hhi1*, *pag1* and *actin* as follows:

hhi1-1F: 5′-CGATATGAACATTAACGATGACGATC-3′;
hhi1-1R: 5′-AAACGGATGCAAAATGGACTCAA-3′;
pag1-F: 5′-ACGGGAATTCAGTGTCGAGGACTT-3′
pag1-R:5′-CATGTCTAGAACCCTACCTACCT-3′
actin-F: 5′-CGTGATGGTGGGCATGGGTCAG-3′;
actin-R:5′-CTAATGTCACGCACGTATTCC-3′.

RNA interference. For *pag1* knockdown experiments, all siRNAs were predicted and synthesized by MDbio Inc. The siRNAs used in this study were *pag1* siRNA (5′-GGAGUAGGUAGCAUAGAUG-3′) and *egfp* siRNA (5′-GGCGAUGCCACCUACGGCAAG-3′). Sf-21 cells (4×10^4) in 96-well plates were transfected with 50 nM of the siRNA using Silencer siRNA transfection kit II (Applied Biosystems). Cells were transfected with designed siRNAs and, at 4 hpt, the cells were infected with *Hz*NV-1 virus (MOI = 1). The number of latently infected colonies was calculated at 12 dpi[4]. Primers designed for the detection of *hhi1* and *pag1* by RT-PCR are listed as following:

hhi1-1F: 5′-CGATATGAACATTAACGATGACGATC-3′
hhi1-1R: 5′-AAACGGATGCAAAATGGACTCAA-3′
pag1-F: 5′-ACGGGAATTCAGTGTCGAGGACTT-3′
pag1-R:5′-CATGTCTAGAACCCTACCTACCT-3′

Construction of *pag1*-null *Hz*NV-1. A fragment containing *egfp* gene flanked by *EcoR*V sites was inserted down-stream of the *hsp 70* promoter (*p-hsp*) of plasmid pBShsp70, generating plasmid pBShE. Fragments containing −50 to −300 nt and +300 to +550 nt of *pag1*, respectively, were obtained by PCR and subsequently cloned into the upstream and downstream, respectively, of *hsp-egfp* of pBShE, yielding pBSphEp. pBSphEp and *Hz*NV-1 viral DNA were co-transfected into Sf-21 cells to generate *pag1*-null *Hz*NV-1 virus. Several primers were designed for the PCR amplification of *pag1* upstream and downstream fragments and they are listed as following:

pag1UF: 5′-ATTGAGCTCCCGAGACTGCACTTTTTAAA-3′
pag1UR: 5′-ATTCCGCCGTAGTAGGTGTGCACGTTTT 3′
pag1DF: 5′-ATTATCGATGTGTATTTATAGATGCCCGA-3′
pag1DR: 5′-ATTATCGATAATACGCAGGGTGGACTCTT-3′

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Author contributions

Guarantors of integrity of entire study, study concepts, and manuscript preparation, Y.C.C.; study design, data acquisition/analysis, literature research and manuscript preparation, Y.L.W.; computational prediction, P.W.C.H.; data acquisition/analysis, manuscript editing, and revision, C.P.W., C.Y.Y.L. and E.C.W. All authors reviewed the manuscript.

Additional information

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